Involvement of MACH, a Novel MORT1/FADD-Interacting Protease, in Fas/APO-1- and TNF Receptor–Induced Cell Death

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Summary

Fas/APO-1 and p55 tumor necrosis factor (TNF) receptor (p55-R) activate cellular mechanisms that result in cell death. Upon activation of these receptors, Fas/APO-1 binds a protein called MORT1 (or FADD) and p55-R binds a protein called TRADD. MORT1 and TRADD can also bind to each other. We have cloned a novel protein, MACH, that binds to MORT1. This protein exists in multiple isoforms, some of which contain a region that has proteolytic activity and shows marked sequence homology to proteases of the ICE/CED-3 family. Cellular expression of the proteolytic MACH isoforms results in cell death. Expression of MACH isoforms that contain an incomplete ICE/CED-3 region provides effective protection against the cytotoxicity induced by Fas/APO-1 or p55-R triggering. These findings suggest that MACH is the most upstream enzymatic component in the Fas/APO-1- and p55-R-induced cell death signaling cascades.

Introduction

Cytotoxicity of lymphocytes is mediated, in part, by interaction of a lymphocyte-produced ligand with Fas/APO-1 (CD95), a widely occurring cell surface receptor belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF) family that can trigger cell death (Nagata and Golstein, 1995). Cell killing by mononuclear phagocytes also involves a ligand–receptor pair, TNF and its p55 receptor (p55-R, CD120a), which are structurally related to the ligand of Fas/APO-1 and Fas/APO-1 (Vandenabeele et al., 1995). Recently, three proteins that bind to the intracellular domains of Fas/APO-1 or p55-R, or both, at the region involved in the receptor-induced death (the death domains) through heteroassociation of homologous regions found within the proteins have been identified using a yeast two-hybrid screen. These proteins, MORT1 (or Fas-associating protein with death domain [FADD]) (Boldin et al., 1995b; Chinnaiyan et al., 1995), which binds specifically to Fas/APO-1, TNFR1-associated death domain protein (TRADD) (Hsu et al., 1995), which binds to p55-R, and receptor-interacting protein (RIP) (Stanger et al., 1995), which binds to both receptors, are also capable of binding to each other (Varfolomeev et al., 1996; Hsu et al., 1996). Cellular expression of MORT1 mutants lacking the N-terminal part of the molecule has been shown to block cytotoxicity induction by Fas/APO-1 or p55-R (Hsu et al., 1996; Chinnaiyan et al., 1996; M. P. B., T. M. G., and D. W., unpublished data), indicating that this N-terminal region transmits the signaling for the cytocidal effect of both receptors through protein–protein interactions.

Recent studies implicate a group of cytoplasmic thiol proteases that are structurally related to the Caenorhabditis elegans protease, CED-3, and the mammalian interleukin-1β (IL-1β)-converting enzyme (ICE) in the onset of various physiological cell death processes (reviewed by Kumar, 1995; Henkart, 1996). There are also indications that these proteases are involved in Fas/APO-1- and TNF-induced cytotoxicity. Specific peptides and virus-encoded proteins that block protease function were found to protect cells from Fas/APO-1- and TNF receptor–mediated cytotoxicity (Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995; Xue and Horvitz, 1995; Beidler et al., 1995). In addition, rapid cleavage of specific intracellular proteins, mediated by protease(s) of the ICE/CED-3 family, was observed shortly after Fas/APO-1 or p55-R stimulation (Tewari et al., 1995a, 1995b; Martin et al., 1995). The mechanisms of activation of the proteases by the receptors have not yet been established. We report the cloning of a novel ICE/CED-3 protease, MACHα, which binds to MORT1; its activity appears to be required for Fas/APO-1- and p55-R-induced cytotoxicity. Our results identify a proteolytic signaling pathway for apoptosis that includes ligand-activated receptors (Fas/APO-1 and p55-R), adapter proteins (MORT1 and TRADD), and their target, MACHα, a proteolytic enzyme.

Results

Two-Hybrid Screening for Proteins That Bind to MORT1 Reveals a Novel Protein That Shares a Sequence Motif with MORT1

Screening of a human B cell cDNA library (Durfee et al., 1993) by the two-hybrid technique for proteins that bind to MORT1 yielded cDNA clones of MORT1 itself, reflecting its ability to self-associate (Boldin et al., 1995b), as well as of TRADD, which binds effectively to MORT1 (Varfolomeev et al., 1996; Hsu et al., 1996). It also yielded clones of a cDNA with a novel sequence. As described below, this protein was later found to occur in multiple isoforms, some of which contain a region homologous to CED-3 and ICE. Following the terminology used for some of the other ICE/CED-3 homologs, we called the protein "MACH" (for MORT1-associated CED-3 homolog; "mach" also means "deteriorating" in Hebrew). For reasons explained below, the isoform cloned in the two-hybrid screen with MORT1 was called "MACHβ1". Deletion analysis showed that MACHβ1 binds to the N-terminal part of MORT1, which is involved in cell death induction (Chinnaiyan et al., 1995). MACHβ1 also self-associated in the transfected yeast. However, it did not bind to several control proteins and, unlike MORT1, was unable to bind to Fas/APO-1 (Figure 1A).

Expression of MACHβ1 molecules in mammalian cells yielded a 34 kDa protein that bound to MORT1 molecules coexpressed with it (Figure 1C). It was also able

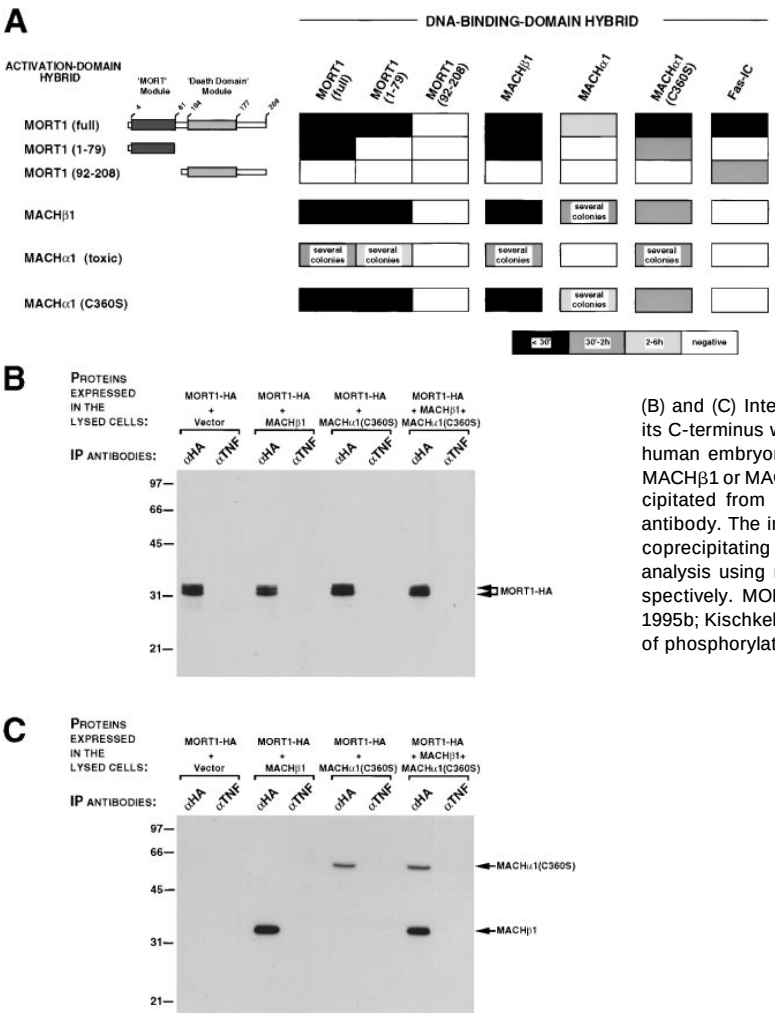

Figure 1. Binding of MACH to MORT1

(A) Interactions within transfected yeast. Interactions of MORT1, its deletion constructs, MACHβ1, and MACHα1, a MACHα1 mutant in which the catalytic cysteine residue (Cys-360) is replaced by Ser (MACHα1(C360S)), and the intracellular domain of human Fas/APO-1 (Fas-IC) within transfected yeast were assessed by a two-hybrid β-galactosidase expression filter assay. The results are expressed as the time required for development of strong color. None of the examined inserts interacted with control proteins, including the intracellular domains of human p55 TNF receptor, p75 TNF receptor, and CD40, and lamin, cyclin D, and empty GAL4 vectors.

(B) and (C) Interactions within mammalian cells. MORT1 fused at its C-terminus with the HA epitope (MORT1–HA) was expressed in human embryonic kidney 293-EBNA cells, alone or together with MACHβ1 or MACHα1(C360S), or both. MORT1–HA was immunoprecipitated from lysates of the cells using an anti-HA monoclonal antibody. The immunoprecipitated MORT1–HA protein (B) and the coprecipitating MACH molecules (C) were detected by Western blot analysis using rabbit anti-MORT1 and anti-MACHβ1 antisera, respectively. MORT1–HA appears as a double band (Boldin et al., 1995b; Kischkel et al., 1995), probably because of differing degrees of phosphorylation of the MORT1 molecules (Kischkel et al., 1995).

to bind to a glutathione-S transferase (GST)–MORT1 fusion protein in vitro (data not shown).

Comparison of the amino acid sequences in MACHβ1 and MORT1 (Figures 2A and 2B) revealed a shared sequence motif, which we called the "MORT module," occurring once in MORT1 and twice in MACHβ1. The same motif is also found in PEA-15, an astrocyte phosphoprotein of unknown function (Figure 2B). This motif is distinct from the death domain motif through which MORT1 binds to Fas/APO-1 (Boldin et al., 1995b; Chinnaiyan et al., 1995). Preliminary data suggest that the MORT motif is involved in the binding of MACHβ1 (and of other MACH isoforms) to MORT1 (data not shown).

MACH Occurs in Multiple Isoforms

Northern blot analysis revealed size heterogeneity of MACH transcripts, ranging between 2.85 kb and 3.5 kb. Both the amounts and the sizes of the transcripts varied among different human tissues (Figure 3A), and were not correlated with the expression of MORT1 (Figure 3B; Chinnaiyan et al., 1995) or of Fas/APO-1 (Watanabe et al., 1992; Leithauser et al., 1993). In the testis and skeletal muscle, for example, MACH transcripts were barely detectable, even though these tissues express significant amounts of MORT1. Conversely, resting peripheral blood mononuclear leukocytes, in which MORT1 expression is very low, were found to express MACH at high levels. Lectin activation of the leukocytes results in a marked change in the size pattern of MACH transcripts, along with an induction of MORT1 (E. Varfolomeev, M. P. B., and D. W., unpublished data).

To determine the structural basis for the size heterogeneity of the MACH transcripts, we screened cDNA libraries for transcripts that hybridize with the MACHβ1 cDNA probe. Using reverse transcriptase–polymerase chain reaction (RT–PCR), we also attempted to clone related transcripts from tumor cell lines. Screening revealed the existence of multiple MACH isoforms, most probably produced by alternative splicing (see Figures 2A and 2C).

All the MACH isoforms shared a common 182 amino acid N-terminal region (which encompasses the MORT modules), but had different C-termini. Three isoforms, defined as subgroup α, have a C-terminus that closely resembles proteases of the ICE/CED-3 family (see below). Four others, defined as subgroup β, have different C-termini owing to alteration in the reading frame. Two (MACHβ1 and MACHβ2) share the C-terminus found in

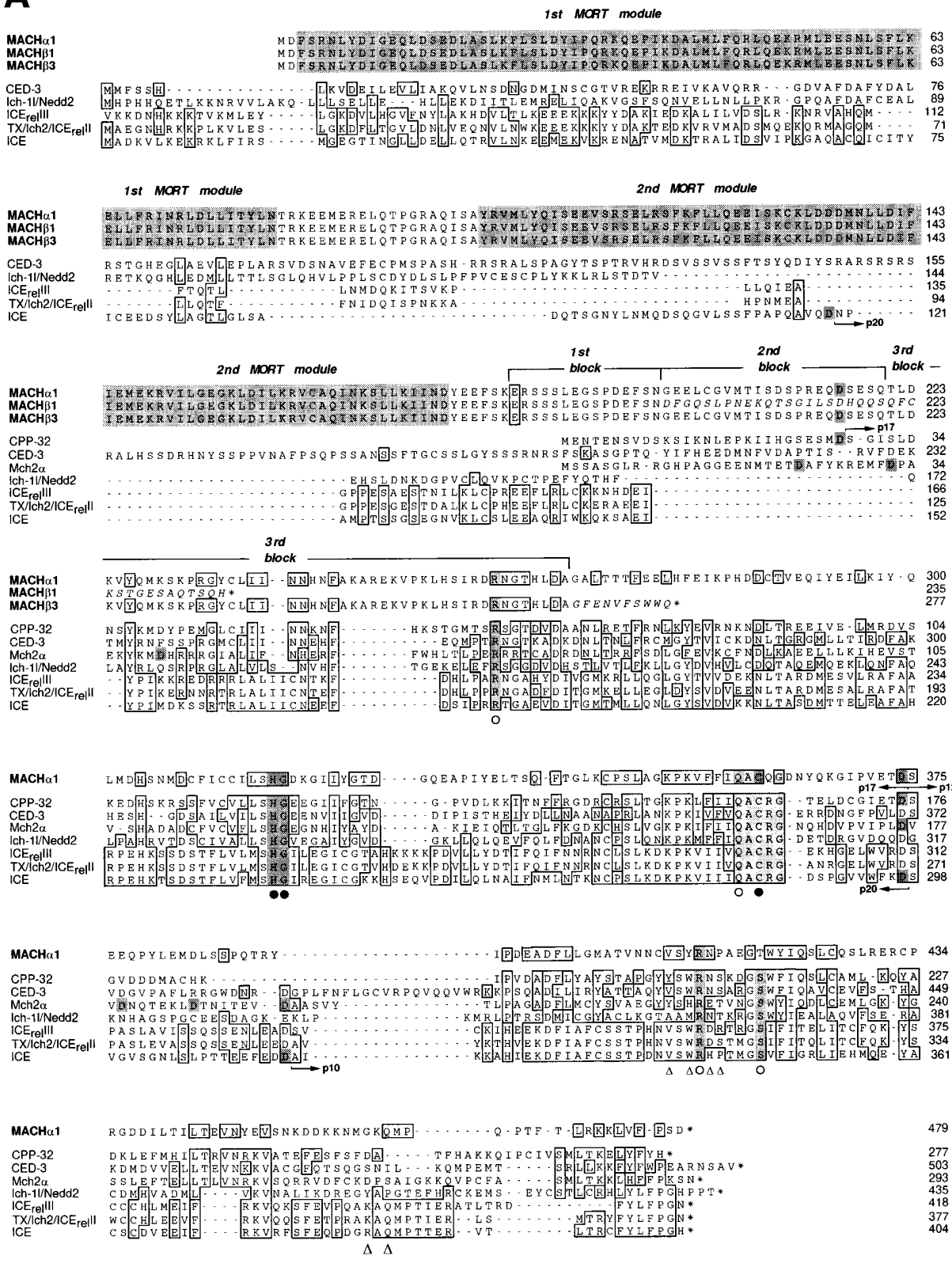

Figure 2. Amino Acid Sequences of the MACH Isoforms, and Conserved Motifs Found in the Proteins

(A) Colinear amino acid sequence alignment of three of the MACH isoforms, CED-3 (Ellis and Horvitz, 1986; Yuan et al., 1993), and the known human proteases of the ICE/CED-3 protease family: CPP32 (Fernandes-Alnemri et al., 1994), also called apopain (Nicholson et al., 1995), and

(*Figure 2 continued on next page*)

B

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MORT1     4   FLVLLHSVSSSLSSSELTELKFLCLGRVGKRKLERVQSG   42
MACH      3   FSRNLYDIGEQLDSEDLASLKFLSLDYIPQRKQEPIKDA   41
MACH    101   YRVMLYQISEEVSRSELRSFKFLLQEEISKCKLDDDMNL   139
PEA-15    4   YGTLFQDLTNNITLEDLEQLKSACKEDIPSEKSEEITTG   42

MORT1    43   LDLFSMLLEQNDLEPGHTELLRELLASLRRHDLLRRVDD   81
MACH     42   LMLFQRLQEKRMLEESNLSFLKELLFRINRLDLLITYLN   80
MACH    140   LDIFIEMEKRVILGEGKLDILKRVCAQINK-SLLKIINDI  177
PEA-15   43   SAWFSFLESHNKLDKDNLSIIEHIFEISRRPDLLTMVVD   81
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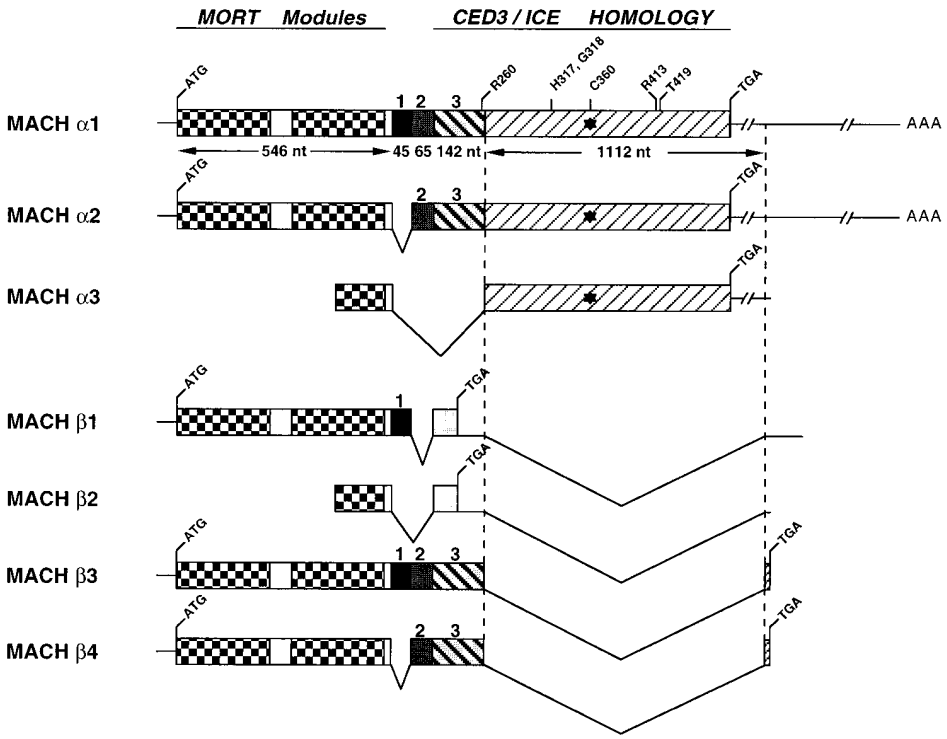
C



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Yama (Tewari et al., 1995b), Mch2α (Fernandes-Alnemri et al., 1995), Ich-1 (Wang et al., 1994) (Ich-1 is the human homolog of the mouse Nedd2 protein, Kumar et al., 1994), ICE rel-III (Munday et al., 1995), ICE rel-II (Munday et al., 1995), also called TX and Ich2 (Faucheu et al., 1995; Kamens et al., 1995), and ICE (Thornberry et al., 1992; Cerretti et al., 1992). Amino acid residues are numbered to the right of each sequence. Dotted lines indicate gaps in the sequence to allow optimal alignment. Amino acids that are identical in at least three members of the ICE/CED-3 protease family shown are boxed. The MORT modules upstream of the ICE/CED-3 homology region are shaded. The three amino acid blocks downstream of the MORT module region (blocks 1–3, see text for explanation) are denoted by overlinings. Within the ICE/CED-3 homology region, amino acids aligning with residues within ICE that were implicated in catalytic activity by X-ray crystallography are denoted as follows: the residues putatively involved in catalysis, corresponding to His-237, Gly-238, and Cys-285 in ICE, are heavily shaded and marked by closed circles below the alignment. The residues constituting the binding pocket for the carboxylate side chain of the P_1 Asp, corresponding to Arg-179, Gln-283, Arg-341, and Ser-347 in ICE, are less heavily shaded and marked by open circles below the alignment. The QACRG motif, which is conserved in all previously described proteases of the ICE/CED-3 family, is lightly shaded. Residues proximal to P_1–P_4 residues of the substrate are marked by triangles below the alignment. Known and suggested Asp-X cleavage sites and potential sites of cleavage found at similar locations in MACH are shaded but not boxed. Arrows indicate the N- and C-terminal ends of the p20 and p10 subunits of ICE and of the p17 and p12 subunits of CPP32. The C-termini of the proteins are denoted by asterisks. Amino acid residues in the unique C-terminal sequences of MACHβ1 and MACHβ3 are written in italics.

(B) The MORT module: sequence homology of the modules in MORT1, MACHβ1 (as well as in the other MACH isoforms), and the PEA-15 gene (GenBank accession number X86809). Identical and similar residues are denoted by boxed and shaded areas, respectively. Sequence identity between the four modules ranged between 18%–38%, and sequence similarity between 47%–56%.

(C) Diagrammatic representation of the various MACH isoforms. Coding regions are represented as boxed areas. The various domains within the coding regions are denoted by different shadings as follows: the MORT modules by the checkered areas; the three amino acid sequence blocks, which occur in different combinations in the isoforms, indicated by numbers as in (A), by the heavily shaded, medium shaded, and the dark striped areas. Positions of the residues in the ICE/CED-3 homology region implicated in the catalytic activity of ICE are shown. The catalytic cysteine residue is indicated by a star. Those parts of the MACHα1 nucleotide sequence that are missing in the sequences of other isoforms are indicated in the diagrams of the latter isoforms by V-shaped connecting lines. The lengths of these cDNA regions, which probably correspond to distinct exons, are indicated below the diagram of MACHα1. Lack of the 65 nt that in MACHα1 encode for block 2 causes alteration in MACHβ1 and MACHβ2 of the reading frame of the nucleotides that encode for block 3. In those isoforms, therefore, these nucleotides encode other amino acids, which together constitute their unique C-terminal region, indicated by the most lightly shaded areas.

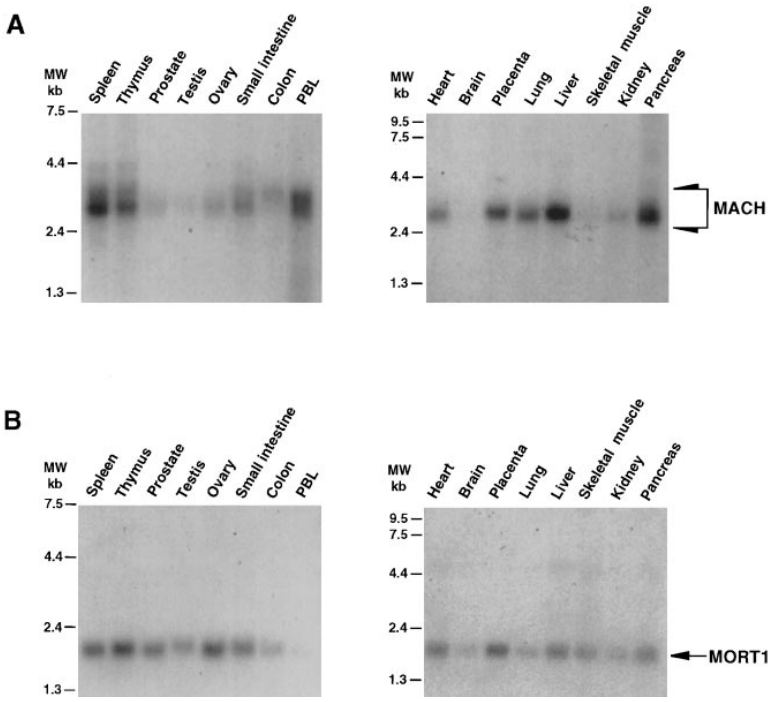


Figure 3. Identification of the MACH Transcripts

Northern blot analysis of poly(A)$^+$ RNA (2 μg per lane) of various human tissues using cDNA probes corresponding in (A) to nucleotides 190–732 in MACHβ1 (a region common to all MACH isoforms cloned), and in (B) to nucleotides 112–852 in MORT1.

the isoform initially cloned by the two-hybrid screen, and two (MACHβ3 and MACHβ4) share a different C-terminus.

The regions between the MORT modules and the sequences defining subgroups α and β varied from one isoform to another. However, close examination showed that these intermediate regions consist of different combinations of the same three amino acid sequence blocks (see Figure 2, blocks 1, 2, and 3).

Some of the MACH Isoforms Contain a ICE/CED-3 Homology Region

A data bank search revealed that the C-terminal part of the MACHα isoforms, including block 3 and the sequence extending downstream of it, closely resembles proteases of the ICE/CED-3 family. Figure 2A presents a sequence comparison of this region in MACH with various known proteins of this family from human sources and the C. elegans CED-3 protein. The region in MACH most closely resembles CPP32, with 41% identity and 62% homology, and CED-3, with 34% identity and 56% homology. It is less similar to ICE, with 28% identity and 50% homology, and its closely related homologs, ICE rel-II and ICE rel-III.

The following two points of similarity seem to be particularly significant:

First, all known proteases of the ICE/CED-3 family cleave proteins at sites defined by the occurrence of Asp at the P_1 position and a small hydrophobic amino acid residue at P_1', whereas other structural features of the substrate, including the nature of the residues at positions P_2–P_4, are less uniform. Accordingly, the active site residues involved in catalysis and in forming the binding pocket for the carboxylate side chain of the P_1 Asp are conserved in these proteases. As shown in Figure 2A, these residues are also conserved in MACHα1. One exception is a conservative change of Ser to Thr at the site corresponding to Ser-347 in ICE. Another slight, yet potentially important, sequence difference between the MACHα isoforms and other members of the protease family is the substitution of Gln for the Arg found in ICE in position 286. In addition, some of the residues in the MACHα isoforms at the sites located near the substrate P_2–P_4 residues differ from those found in other ICE/CED-3 proteins.

Second, proteases of the ICE/CED-3 family contain sites of cleavage by proteases of this family. Indeed, several of the proteases are known to be self-processed, and depend upon this processing for maximal catalytic activity. Their fully bioactive form is composed of two noncovalently associated cleavage products that differ in size (p20 and p10 in ICE; p17 and p12 in CPP32, marked by arrows in Figure 2A). The presence of potentially autocleavable sites in other members of the family suggests that they are also subject to processing. Such sites occur in MACHα1 at almost the same locations as in CPP32. The site in MACHα1 corresponding to the N-terminus of the p17 subunit of CPP32 is located in

On the other hand, in MACHβ3 and MACHβ4 the reading frame of block 3 is maintained, but absence of the nucleotides that encode the ICE/CED-3 region and part of the 3′ noncoding region results in alteration of the reading frame of nucleotides further downstream. Because of this alteration, the most 5′ part of this noncoding downstream region does encode 10 amino acids, which constitute the C-terminal region unique to these two isoforms, indicated by the most light striped areas. As indicated in the figure, only partial cDNA clones of MACHα3 and MACHβ2 were obtained.

the second conserved amino acid block, just a few amino acids upstream of the N-terminus of the ICE/CED-3 homology region (below Asp-216). As with all other members of the ICE/CED-3 family known to be cleaved, the site in MACHα1 corresponding to the point of cleavage between the two subunits of CPP32 is located a few amino acids downstream of the catalytic cysteine residue (below Asp-374).

MACHα1 Binds to MORT1 and to MACHβ1

To determine whether MACHα1 can bind to MORT1 as MACHβ1 does, we first examined the interaction of the proteins within transfected yeast. As shown below, MACHα1 was cytotoxic to mammalian cells. It was also cytotoxic to the yeast, particularly when expressed in the activation domain (AD) vector (the expression level of which is higher than that of the DNA-binding domain [DBD] vector). On the other hand, MACHα1 in which the catalytic cysteine residue, Cys-360, was replaced with Ser (MACHα1(C360S)) was not cytotoxic to either mammalian cells (see below) or yeast. Like MACHβ1, MACHα1(C360S) bound in transfected yeast to MORT1 and also to itself. It also bound to MACHβ1. Also, yeast expressing the wild-type MACHα1 together with MORT1 or MACHβ1 exhibited interaction of the transfected proteins. The intensity of the lacZ product color varied, however, among the yeast colonies; in yeasts transfected with MACHα1 in both the AD and the DBD vectors, no color product was observed, probably because of the cytotoxic effect of the wild-type MACHα1 (see Figure 1A).

Both MACHα1(C360S) and MACHβ1 coimmunoprecipitated with MORT1 from lysates of human embryonic kidney 293-Epstein–Barr virus nuclear antigen (EBNA) cells, indicating that they bind to MORT1 also in mammalian cells (see Figure 1C).

The ICE/CED-3 Homology Region in MACH Has Proteolytic Activity

Lysates of Escherichia coli in which we had expressed the MACHα ICE/CED-3 homology region in fusion with GST were examined for their ability to cleave fluorogenic peptide substrates, previously shown to be cleaved by other ICE/CED-3 homologs (Figure 4). The lysates effectively cleaved acetyl-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-AMC), whose sequence corresponds to a site in the nuclear protein poly(ADP-ribose) polymerase (PARP), which is cleaved in cells following Fas/APO-1 stimulation (Tewari et al., 1995b) as well as in other apoptotic processes (Kaufmann, 1989; Kaufmann et al., 1993; Lazebnik et al., 1994). This activity was blocked by iodoacetic acid (5 mM), confirming that it is mediated by a thiol protease. No cleavage was observed with lysates containing the GST-fused MACH ICE/CED-3 homology region in which the catalytic cysteine residue Cys-360 was replaced by Ser. Also, lysates from bacteria that expressed the full-length MACHα1 protein as a GST fusion protein did not cleave Ac-DEVD-AMC, probably because of the absence of bacterial enzymes capable of processing the full-length molecule. Nor did cleavage occur with lysates containing either of the two potential cleavage products of the ICE/CED-3 homology region.

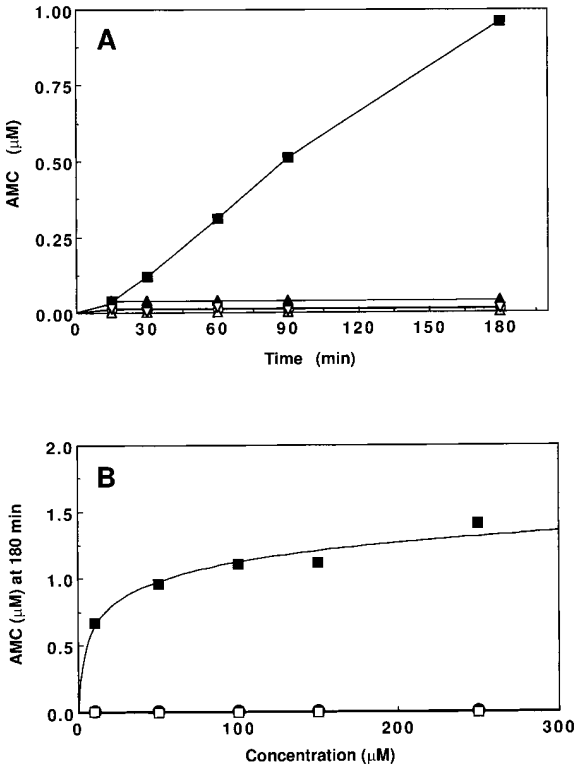


Figure 4. Protease Activity of the ICE/CED-3 Homology Region in MACHα

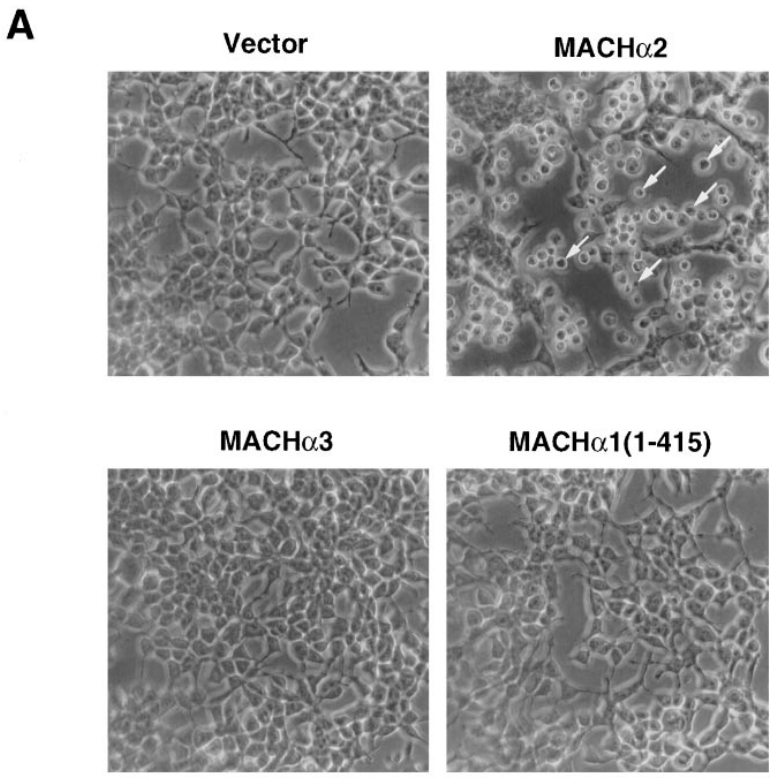
(A) Kinetics of cleavage of the PARP sequence–derived fluorogenic substrate, Ac-DEVD-AMC (50 μM), by extracts of E. coli expressing a GST fusion protein of the ICE/CED-3 homology region in MACHα1 (Ser-217 through the C-terminus of the protein, indicated by the filled boxes) as compared with the lack of cleavage by extracts of bacteria expressing GST fusion products of the full-length MACHα1, indicated by open circles, or of the ICE/CED-3 homology region in which Cys-360 was replaced by Ser, indicated by inverted open triangles, or by extracts of bacteria expressing GST fusion products of either of the two potential proteolytic products of the ICE/CED-3 homology region (Ser-217 through Asp-374, indicated by open triangles, and Ser-375 through Asp-479, the C-terminus of the protein, indicated by filled-in triangles).

(B) Substrate concentration dependence of the cleavage of Ac-DEVD-AMC. The substrate was incubated for 180 min with extracts of bacteria expressing the GST fusion product of the MACHα1 ICE/CED-3 homology region, indicated by filled-in boxes. Cleavage of this substrate by the extracts was inhibited in the presence of iodoacetic acid (5 mM, indicated by open boxes). Ac-YVAD-AMC, a fluorogenic substrate corresponding to an ICE cleavage site in the IL-1β precursor, was not cleaved (indicated by filled-in circles).

None of the tested lysates showed proteolytic activity towards acetyl-Tyr-Val-Ala-Asp-α-(4-methyl-coumaryl-7-amide) (Ac-YVAD-AMC), an ICE cleavage site in the IL-1β precursor (Thornberry et al., 1992).

MACH Molecules That Contain the ICE/CED-3 Homology Region Can Mediate Cell Death

Embryonic kidney 293-EBNA and breast carcinoma MCF7 cells transfected with an expression vector of



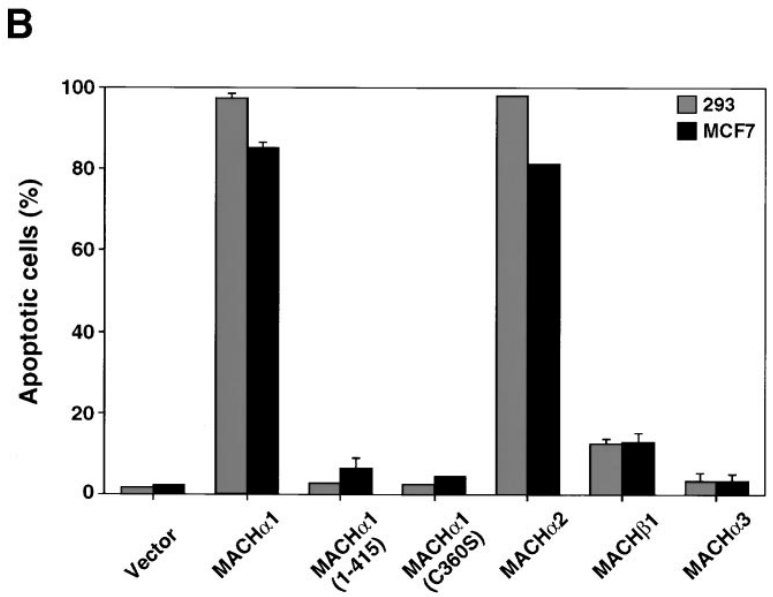


Figure 5. Cell Death Mediation by MACHα1 and MACHα2

(A) Morphology of human embryonic kidney 293-EBNA cells transiently expressing the indicated MACH isoforms. The arrows point to apoptotic cells. Photographs were taken 26 hr after transfection.

(B) Quantification of MACH-induced death of the 293-EBNA and MCF7 cells by determination of the portion of β-galactosidase-expressing cells exhibiting apoptotic morphology 20 hr after transfection of the indicated constructs. Data are from three independent experiments with the 293-EBNA cells and two independent experiments with the MCF7 cells. They are expressed as the mean percentage of the blue cells exhibiting signs of apoptosis as a fraction of the total number of blue cells counted (about 500 cells per sample). The expression construct of MACHα3 used in the experiments presented in this figure and in Figure 6 was created by reconstituting the missing N-terminal part of this isoform with the corresponding part of MACHα1.

either MACHα1 or MACHα2 exhibited massive cell death, manifested by cell rounding, blebbing, contraction, and finally detachment of cells from the dish (Figure 5A, top; data not shown). By 20 hr after transfection, most of the transfected cells exhibiting β-galactosidase staining (used as a transfection marker) showed condensed morphology typical of apoptosis (Figure 5B).

No death (beyond the slight amount observed in control cells transfected with an empty expression vector) occurred in 293-EBNA or MCF7 cells transfected with the expression vectors for MACHα3, MACHα1(1–415), or MACHα1(C360S) (Figure 5). Cells transfected with MACHα1 together with these vectors also exhibited very little cell death, indicating that MACH molecules with an incomplete ICE/CED-3 region have a negative dominant effect on the activity of the wild-type molecules (data not shown). Cultures expressing MACHβ1, which lacks the ICE/CED-3 region, exhibited some slight cell death (Figure 5B). In HeLa cells, this effect of MACHβ1 was, for some reason, more pronounced, and also MACHα3, MACHα1(1–415), and MACHα1(C360S) were somewhat cytotoxic (Figures 6C and 6D; data not shown).

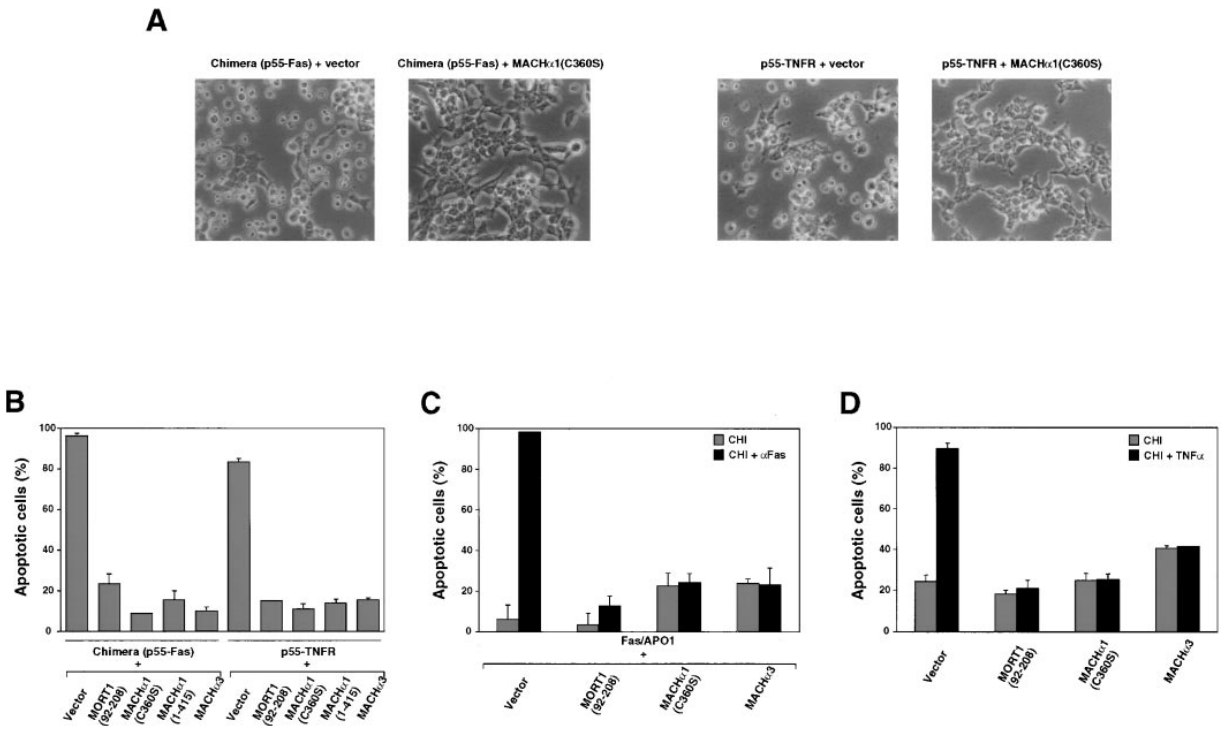


Figure 6. MACHα Molecules That Contain a Nonfunctional ICE/CED-3 Region Block Cell Death Induction by Fas/APO-1 and p55-R

(A) Morphology of 293-EBNA cells in which cell death was induced by transient expression of a chimera comprised of the extracellular domain of the p55-R (amino acids 1–168) fused to the transmembrane and intracellular domains of Fas/APO-1 (amino acids 153–319) (p55-Fas chimera), or by expression of the p55-R, and of cells that were protected from these cytotoxic effects by their simultaneous transfection with MACHα1(C360S). Photographs were taken 26 hr after transfection.

(B) Quantification of death induced in 293-EBNA cells by their transfection with p55-Fas chimera or with p55-R, together with an empty vector, a MORT1 deletion mutant lacking the MACH-binding region (MORT1(92–208)), or MACHα molecules containing a nonfunctional ICE/CED-3 region.

(C) Death of HeLa cells that transiently express Fas/APO-1, induced by treatment with anti-Fas/APO-1 antibody and cycloheximide, and its prevention by cotransfection of MORT1(92–208), MACHα1(C360S), or MACHα3.

(D) Death of HeLa cells induced by treatment with TNF and cycloheximide, and its prevention as in (C). Data are from at least two independent experiments and are expressed as in Figure 5.

Blocking of MACHα Function Interferes with Cell Death Induction by Fas/APO-1 and p55-R

To assess the contribution of MACHα to Fas/APO-1 and p55-R cytotoxicity, we expressed MACHα3, as well as the nonfunctional MACHα1 mutants, MACHα1(1–415) and MACHα1(C360S), in cells that were induced to exhibit this cytotoxicity. p55-R-induced cytotoxicity was triggered in 293-EBNA cells by transient overexpression of this receptor (Boldin et al., 1995a), and Fas/APO-1 cytotoxicity by overexpression of chimeric molecules comprising the extracellular domain of the p55-R and the transmembrane and intracellular domains of Fas/APO-1. The chimera was considerably more cytotoxic than the normal Fas/APO-1 (data not shown). We also induced cytotoxic activity in HeLa cells by treating them with TNF or anti-Fas/APO-1 antibody in the presence of the protein synthesis blocker cycloheximide. In all systems examined, MACHα3 and the nonfunctional MACHα1 mutants provided effective protection against the receptor-induced cytotoxicity (Figure 6). Such protection was also observed, as previously reported (Hsu et al., 1996; Chinnaiyan et al., 1996), in cells transfected with a MORT1 N-terminal deletion mutant lacking the MACH-binding region. These protective effects indicate that MACHα is a necessary component of both the Fas/APO-1- and the p55-R-induced signaling cascades for cell death.

Discussion

Like other receptor-induced effects, cell death induction by the TNF receptors and Fas/APO-1 occurs via a series of protein–protein interactions, leading from ligand–receptor binding to the eventual activation of enzymatic effector functions, which in the case of these particular receptors results in cell death. Previous studies have elucidated nonenzymatic protein–protein interactions that initiate signaling for cell death: binding of trimeric TNF or the Fas/APO-1 ligand molecules to the receptors, the resulting interactions of their intracellular domains (Brakebusch et al., 1992; Tartaglia et al., 1993; Itoh and Nagata, 1993) (augmented by a propensity of the death domain motifs to self-associate) (Boldin et al., 1995a), and induced binding of two cytoplasmic proteins (which can also bind to each other) to the intracellular domains of the receptors: MORT1 (or FADD) to Fas/APO-1 (Boldin et al., 1995b; Chinnaiyan et al., 1995, Kischkel et al., 1995) and TRADD to p55-R (Hsu et al., 1995, 1996).

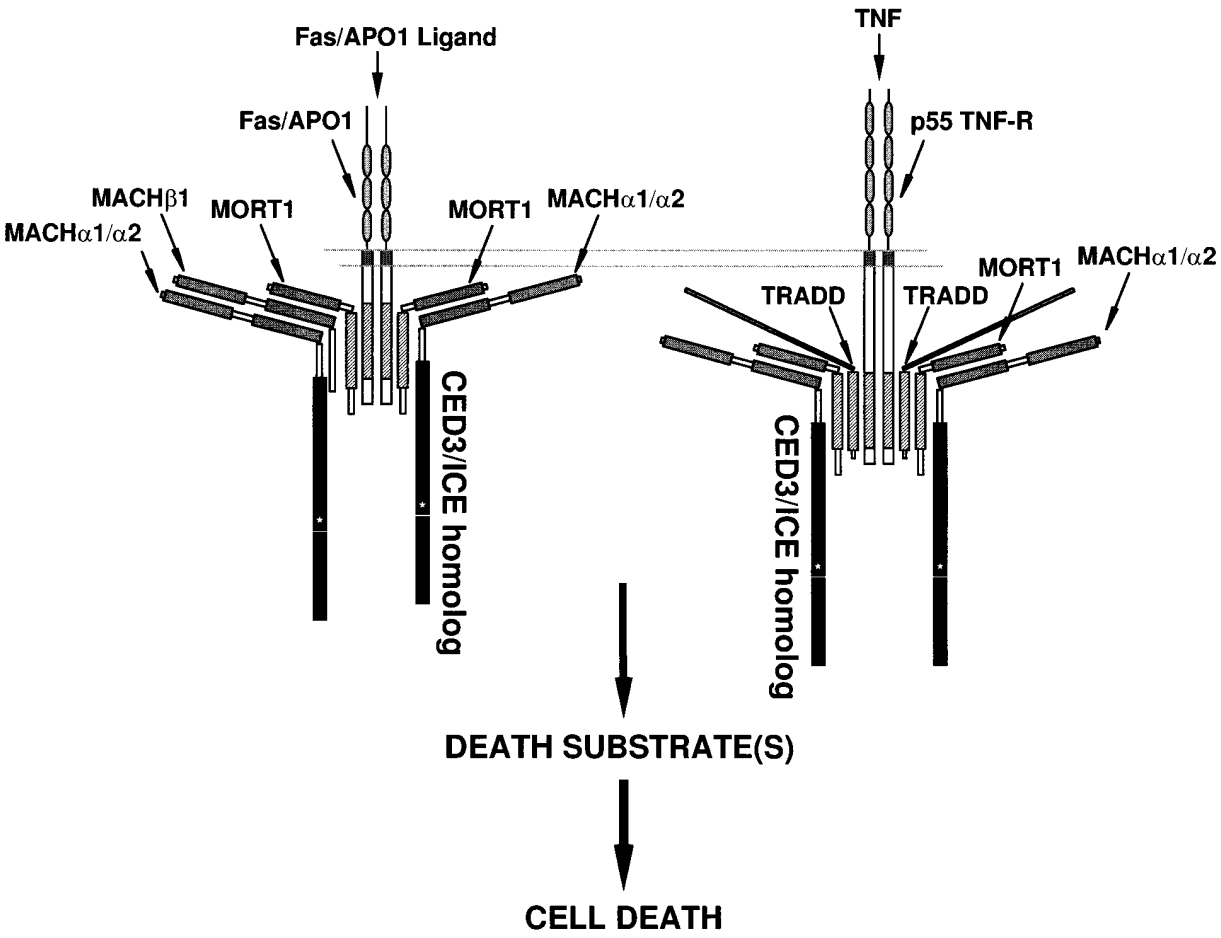


Figure 7. Diagrammatic Representation of the Receptor and Target Protein Interactions Participating in Induction of Cell Death by Fas/APO-1 and p55-R

Death domain module is indicated by striped areas; MORT module is indicated by lightly shaded areas; ICE/CED-3 homology region is indicated by heavily shaded areas.

Here, we describe a protease of the ICE/CED-3 family, MACHα, that binds to MORT1 and initiates cell death. MACHα activity appears to constitute the most upstream enzymatic step in the cascade of signaling for the cytocidal effects of Fas/APO-1 and p55-R (Figure 7).

The regulatory mechanisms of MACHα activity remain to be clarified. Other ICE/CED-3 proteases were shown to exhibit full activity only after proteolytic processing, which occurs either by self-cleavage or via their cleavage by other proteases (reviewed by Kumar, 1995; Henkart, 1996). Our observation that lysates of bacteria that express GST–MACHα1 molecules do not possess enzymatic activity, as opposed to the activity observed in lysates of bacteria that express the ICE/CED-3 homology region, suggests that processing is also required for MACHα activity. The way in which MACHα processing occurs within the mammalian cell, and how this processing is brought about by Fas/APO-1 or p55-R triggering, is not known. MORT1 has been shown to bind in cells to activated Fas/APO-1 together with some other proteins (Kischkel et al., 1995). These proteins are likely to include MACHα1 and other MACH isoforms. It seems plausible that the binding of MORT1 in association with MACHα to Fas/APO-1 brings together several MACH molecules, or induces conformational changes in them, and that these changes either trigger autolytic processing of MACHα or make MACHα susceptible to cleavage by other proteases. Stimulation of p55-R may trigger self-processing of MACHα in a similar, though less direct manner, by bringing together several TRADD molecules, or inducing a conformational change in them, which, in turn, induces a change in the conformation or state of aggregation of MORT1 and its associated MACH molecule.

The substrate specificity of MACHα seems to be rather death oriented. Although it could cleave a substrate peptide corresponding to a cleavage site in the death substrate PARP (Ac-DEVD-AMC), MACHα showed no proteolytic activity towards a peptide corresponding to the site of processing of the IL-1β precursor by ICE (Ac-YVAD-AMC). Identification of the cellular proteins that serve as substrates for cleavage by MACHα will elucidate the more downstream events in death induction by this protease. Likely substrates for MACHα cleavage are other members of the ICE/CED-3 family, such as CPP32 and ICE. Some of these proteases are

indeed processed after Fas/APO-1 or TNF receptor triggering (Miura et al., 1995; Schlegel et al., 1996; Chinnaiyan et al., 1996). Perhaps, proteases that do not belong to the ICE/CED-3 family are also activated by MACHα, either directly or through the action of other ICE/CED-3 proteases. Involvement of multiple proteases in the cell death process is consistent with the reported ability of inhibitors of various proteases, including inhibitors of serine proteases and an inhibitor of ICE cleavage as well as antisense ICE cDNA, to protect cells from Fas/APO-1- and TNF receptor–induced toxicity (Weitzen and Granger, 1980; Ruggiero et al., 1987; Enari et al., 1995; Los et al., 1995).

A variety of other enzymes, including phospholipases, sphyngomyelinases, and protein kinases, may participate in cell death induction by the TNF receptors and Fas/APO-1 (see, e.g., Eischen et al., 1994; Vandenabeele et al., 1995; Cifone et al., 1995, and references therein). Some of these enzymes may become activated by the proteolytic cleavage initiated by MACHα. It also seems possible, however, that at least part of these other death-related activities are stimulated by distinct signaling routes, independently of MACHα stimulation. Involvement of more than one signaling cascade in the induction of cell death, some common to p55-R and Fas/APO-1 and some induced by only one of them, would be consistent with reports on both shared and distinct features of cell death processes induced by the two receptors (Grell et al., 1994; Schulze-Osthoff et al., 1994; Wong and Goeddel, 1994; Clement and Stamenkovic, 1994).

MACH is expressed in different tissues at markedly different levels and apparently also with different isotype patterns. These differences probably contribute to the tissue-specific features of response to the Fas/APO-1 ligand and TNF. As in the case of other ICE/CED-3 homologs (Wang et al., 1994; Alnemri et al., 1995), MACH isoforms containing incomplete ICE/CED-3 regions (e.g., MACHα3) are found to inhibit the activities of coexpressed MACHα1 or MACHα2 molecules; they are also found to block death induction by Fas/APO-1 and p55-R. Expression of such inhibitory isoforms in cells may constitute a mechanism of cellular self-protection against Fas/APO-1- and TNF-mediated cytotoxicity. The wide heterogeneity of MACH isoforms, which greatly exceeds that observed for any of the other proteases of the ICE/CED-3 family, should allow a particularly fine tuning of the function of the active MACH isoforms.

It is possible that some of the MACH isoforms serve additional functions. The ability of MACHβ1 to bind to both MORT1 and MACHα1 suggests that this isoform might enhance the activity of the enzymatically active isoforms. The mild cytotoxicity observed in 293-EBNA and MCF7 cultures transfected with this isoform and the rather significant cytotoxic effect that it exerts in HeLa cells probably reflect activation of endogenously expressed MACHα molecules upon binding to the transfected MACHβ1 molecules. Conceivably, some of the MACH isoforms could also act as docking sites for molecules that are involved in other noncytotoxic effects of Fas/APO-1 and TNF receptors.

Owing to the unique ability of Fas/APO-1 and TNF receptors to cause cell death, as well as the ability of the TNF receptors to trigger other tissue-damaging activities, aberrations in the function of these receptors could be particularly deleterious to the organism. Indeed, both excessive and deficient functioning of these receptors have been shown to contribute to pathological manifestations of various diseases (Vassalli, 1992; Nagata and Golstein, 1995). Identifying the molecules that participate in the signaling activity of the receptors, and finding ways to modulate the activity of these molecules, could direct new therapeutic approaches. In view of the suspected central role of MACHα in Fas/APO-1- and TNF-mediated toxicity, it seems particularly important to design drugs that can block the proteolytic function of MACHα, as was done for some other proteins of the ICE/CED-3 family. The unique sequence features of the ICE/CED-3 homolog within MACHα molecules could permit the design of drugs that would specifically affect its activity. Such drugs could provide protection from excessive immune-mediated cytotoxicity involving MACHα, without interfering with the physiological cell death processes in which other members of the ICE/CED-3 family are involved.

Experimental Procedures

Cloning of MACHβ1 and Study of Its Binding Properties by Two-Hybrid β-Galactosidase Expression Test

MACHβ1 was cloned by two-hybrid screening (Fields and Song, 1989) of a GAL4 AD-tagged human B cell library (Durfee et al., 1993) for proteins that bind to MORT1, using the HF7c yeast reporter strain (Clontech, Palo Alto, CA). Screening was performed according to the Matchmaker Two-Hybrid System Protocol (Clontech) in the presence of 5 mM 3-aminotriazole. The binding properties of MACHβ1, as well as of other examined proteins, were assessed in the yeast SFY526 reporter strain (Clontech), using the pGBT9 GAL4-DBD and pGAD GH GAL4-AD vectors. Quantification of the binding in yeast by the β-galactosidase expression filter assay was performed as described (Boldin et al., 1995b).

Cloning of Other MACH Isoforms

MACHα1 and MACHα2 were cloned from a Charon BS cDNA library derived from the mRNA of human thymus (donated by P. Sankhavaram, Yale University). The library was screened under stringent conditions with a MACHβ1 cDNA probe, labeled using a random-priming kit (Boehringer Mannheim). The other MACH isoforms were cloned by RT–PCR, performed on total RNA from Raji (MACHα1, MACHα2, MACHα3, MACHβ3, and MACHβ4) and Daudi (MACHα2, MACHβ2, MACHβ3, and MACHβ4) human lymphoblastoid cells. Reverse transcriptase reaction was performed with an oligo(dT) adapter primer (5′-*GACTCGAGTCTAGAGTCGAC(T)*$_{17}$–3′) and the SuperScript II reverse transcriptase (GIBCO BRL), used according to the instructions of the manufacturer. The first round of PCR was performed with the Expand Long Template PCR System (Boehringer Mannheim), using the following sense and antisense primers: 5′-*AAGTGAGCAGATCAGAATTGAG*-3′, corresponding to nucleotides 521–542 of the MACHβ1 cDNA, and 5′-*GACTCGAGTCTAGAG TCGAC*-3′, respectively. The second round was performed with Vent polymerase (New England Biolabs) using the following sense and antisense nested primers: 5′-*GAGGATCCCCAAATGCAAACTGGAT GATGAC*-3′ and 5′-*GCCACCAGCTAAAAACATTCTCAA*-3′, which correspond to nucleotides 575–597 and nucleotides 930–953 of MACHβ1 cDNA, respectively. To confirm that MACHβ3 and MACHβ4 have initiation codons, we cloned a more 5′ sequence of these isoforms from the RNA of Raji cells. The RT–PCR reaction, performed using the oligo(dT) adapter primer as described above, was followed by two rounds of PCR (with Vent polymerase) (NEB), using the following sense and antisense oligonucleotides: 5′-*TTGGATCCAGATGGACTTCAGCAGAAATCTT*-3′ and 5′-*ATTCT CAAACCCTGCATCCAAGTG*-3′, which correspond to nucleotides

188–210 and nucleotides 914–937 in MACHβ1, respectively. (The latter oligonucleotide is specific to the β isoforms.) Among the clones obtained in this way, those found to contain the nucleotides encoding for the amino acids of block 2 (whose presence distinguishes MACHβ3 and MACHβ4 from MACHβ1 and MACHβ2; see legend for Figure 2C) were fully sequenced. Nucleotide sequences in all cloned isoforms were determined in both directions by the dideoxy-chain termination method. Only partial cDNA clones of MACHα3 and MACHβ2 were obtained.

Northern Blot Analysis

cDNA probes were radiolabeled with the random-prime kit (Boehringer Mannheim) and applied for analysis of human multiple tissue blots (Clontech) according to the instructions of the manufacturer.

Expression Vectors

All cDNA sequences used were of human origin. The deletion mutants, the GST fusions, and the p55-R-Fas chimera were produced by PCR or conventional cloning techniques, or both, and the point mutants by oligonucleotide-directed mutagenesis. Proteins of the Fas/APO-1 or p55-R signaling cascades were expressed in mammalian cells using the pcDNA3 expression vector (Invitrogen). β-galactosidase was expressed using the pSV–β-gal vector (Promega).

Antibodies

Rabbit anti-MACHβ1 and anti-MORT1 antisera were raised against GST–MACHβ1 and GST–MORT1 fusion proteins. Mouse anti-Fas/APO-1 monoclonal antibody, CH11, was purchased from Oncor (Gaithersburg, MD). Mouse monoclonal anti-HA epitope antibody (12CA5) (Field et al., 1988) and anti-TNFα antibody were produced in our laboratory.

Immunoprecipitation and Western Blot Analysis

Human embryonic kidney 293-EBNA cells (Invitrogen) were transfected in aliquots of 4×10^6 (2×10^6 per dish [10 cm], 20 μg of DNA per dish) by the calcium phosphate method. We lysed the cells 26 hr after transfection in buffer containing 50 mM HEPES (pH 7.5), 250 mM NaCl, 0.2% NP-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin (lysis buffer). Immunoprecipitation was performed by incubation (2 hr at 4°C) of aliquots (1 ml) of lysate (2×10^6 cells per aliquot) with anti-HA epitope antibody or as a control, with a monoclonal antibody against TNFα (5 μg/aliquot) and with protein G–agarose beads (30 μl/aliquot). Immunoprecipitates were washed three times with lysis buffer and once with phosphate-buffered saline, fractionated by 12% SDS–PAGE, and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Western blot analysis was performed with rabbit anti-MORT1 and anti-MACHβ1 antisera, applied at a dilution of 1:1000, and the ECL kit (Amersham, Buckinghamshire, England).

Protease Activity Assays

GST fusion proteins of MACHα1, its ICE/CED-3 homology region, the homology region in which Cys-360 was replaced by Ser, and the potential cleavage products of this region were produced in XL1-blue bacteria using the pGEX3X expression vector (Pharmacia). Bacteria were lysed by sonication in extraction buffer containing 25 mM HEPES (pH 7.5), 0.1% 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate, 5 mM EDTA, and 2 mM DTT, followed by centrifugation at 16,000 × g for 10 min. SDS–PAGE analysis confirmed the presence of similar levels of the various fusion proteins in the lysates (data not shown). Aliquots (50 μl) of the bacterial extracts (200 μg protein) were diluted 10-fold in extraction buffer containing the indicated concentrations of the fluorogenic substrates, and incubated at room temperature for the indicated periods. Amino-4-methylcoumarin (AMC) release was measured by spectrofluorometry at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. AMC concentration was determined from a standard curve. Both fluorogenic substrate peptides were obtained from Peptide Institute, Incorporated (Osaka, Japan).

Cell Death Assays

293-EBNA, MCF7 human, and HeLa cells (clone HtTA-1, obtained from Dr. H. Bujard) were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells (5×10^5 293-EBNA cells, 3×10^5 MCF7 cells, or 3×10^5 HeLa cells in dishes [6 cm]) were transiently transfected, using the calcium phosphate precipitation method, with the cDNAs of the indicated proteins together with the β-galactosidase expression vector. In the experiments presented in Figure 5, each dish was transfected with 3.5 μg of the indicated MACH construct and 1.5 μg of pSV–β-gal. In the experiments presented in Figures 6A, 6B, and 6C, each dish was transfected with 2.5 μg of the p55-R, p55-Fas chimera (for 6A and 6B), or Fas/APO-1 (for 6C) constructs, 2.5 μg of the indicated MACH or MORT1 construct (or, as control, empty vector), and 1.5 μg of pSV–β-gal. In the experiment of Figure 6D, each dish was transfected with 5 μg of the indicated MACH or MORT1 construct (or, as control, empty vector) and 1.5 μg of pSV–β-gal. Cells were rinsed 6–10 hr after transfection. The 293-EBNA and MCF7 cells were incubated for a further 18 hr without additional treatment. The HeLa cells were incubated for 26 hr after transfection and then for 5 hr in the presence of either anti-Fas/APO-1 antibody (CH11, 0.5 μg/ml) or TNF (100 ng/ml), together with cycloheximide (10 μg/ml). The extent of cell death at the end of the incubation periods was assessed by determination of β-galactosidase expression, as described by Kumar et al. (1994).

Acknowledgments

Correspondence should be addressed to D. W. We are grateful to Dr. Yosef Yarden for critical reading of the manuscript. We also thank Drs. Stephen J. Elledge and Pat Sankhavaram for cDNA libraries, Dr. Jacques H. Camonis for advice in applying the two-hybrid system technique, Dr. Rivka Adar for skillful creation of the MACHα1 mutants and assistance in the two-hybrid tests, Dr. Batya Cohen for helpful suggestions on RT–PCR, Dr. Igor Mett for advice on cell death quantitation, Dr. Shmaria Blumberg for the gift of AMC, Andreas Schwarz and Sweta Boldin for assistance in photographing cells, and Dr. Doron Lancet and Eugene Varfolomeev for helpful suggestions. This work was supported in part by grants from Inter-Lab, Limited, Ness Ziona, Israel, from Ares Trading S. A., Switzerland, and from the Israeli Ministry of Arts and Sciences.

Received January 19, 1996; revised May 13, 1996.

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GenBank Accession Numbers

The accession numbers for the cDNA sequences of the MACH isoforms reported in this paper are X98172 to X98178.